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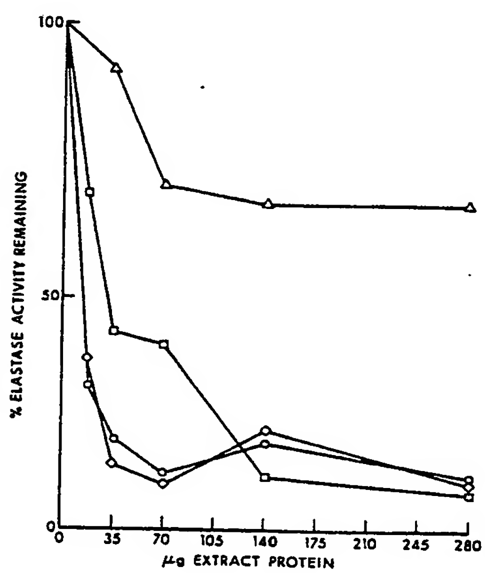
(54) Title: HUMAN LEUKOCYTE ELASTASE INHIBITORS PREPARED FROM PYOGENIC BACTERIA AND METHODS FOR THEIR PURIFICATION

(57) Abstract

Proteinaceous materials extracted from pyogenic bacteria, streptococci, staphylococci and Klebsiella species, act as specific inhibitors of human luekocyte elastase and may be used to treat deseases which include elastolysis as part of their desease processes e.g., pulmonary emphysema, adult respiratory distress syndrome and bacterial pneumonites. The proteinaceous materials extracted from the pyogenic bacteria can be conveniently purified by providing an affinity column by various novel methods such as the following five step methods: (1) binding elastin to an affinity column support material (2) binding human leukocyte elastase to the elastin (3) binding the human leukocyte elastase inhibitors to the elastase (4) eluting the affinity column with a first elution medium capable of separating contaminating substances from the inhibitors and (5) separating the inhibitors from the elastase by use of a second elution medium having a raised salt concentration.



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# HUMAN LEUKOCYTE ELASTASE INHIBITORS PREPARED FROM PYOGENIC BACTERIA AND METHODS FOR THEIR PURIFICATION

## TECHNICAL FIELD OF THE INVENTION

This invention relates generally to substances for treating diseases which include elastin destruction as part of the disease process. More specifically this invention is concerned with proteinaceous substances which inhibit the elastolytic action of human leukocyte elastase and with methods for purifying human leukocyte elastase inhibitors.

## BACKGROUND ART

Neutrophil proteases have been implicated in several human diseases since the turn of the century (Metchnikoff, E. L' Immunite' dans le Maladies Infectieuses, Masson, Paris (1901)). A number of such proteases are known. This patent disclosure is particularly concerned with a class of protease found in normal, circulating human polymorphonuclear leukocytes known for its elastolytic activity, see Janoff, A. Scherer, V. J. Exp. Med. 128:1137-1151 (1968). This class of protease is commonly referred to as human leukocyte elastase ("HLE"), or alternatively, granulocyte elastase or neutrophil elastase, (for the purposes of this patent disclosure, the term HLE should be taken to include all three terms). HLE has been implicated in a wide variety of disease processes which produce elastolytic effects, e.g., pulmonary emphysema (Janoff, A., et al Am. Rev. Respir. Dis. 115:461-478, (1977) and Snider, G.L. Med. Clin. North Am. 65:647-665, (1981)), rheumatoid arthritis and other arthritides involving acute inflammatory reactions (Janoff, A. et al J. Clin. Invest. 57:615-624 (1976) and Keiser, H. et al J. Clin. Invest. 57:625-632 (1976)) and Adult Respiratory Distress Syndrome (Lee, C.T. et al N. Engl. J. Med. 304:192-196 (1981) and McGuire, W.W. et al J. Clin. Invest. 69:543-553 (1982)).

The major tissue regulator of HLE in humans is alpha 1-proteinase inhibitor or alpha 1-antitrypsin. The alpha 1-proteinase inhibitor is often referred to as A1Pi (Ohlsson, K.:Proteases and Biological Control (eds. E. Reich, D. Rifkin, E. Shaw) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 591-602 (1975)). When the normal A1Pi/HLE balance is upset, elastin is destroyed. For example, it has been shown

that a genetic deficiency of A1Pi is associated with markedly increased risk of developing pulmonary emphysema (Eriksson, S. Acta. Med. Scand. 177:(suppl.432)5-12 (1965)). Several other investigators have also suggested that oxidants in cigarette smoke can inactivate A1Pi (Carp, H. and Janoff, A. Am. Rev. Respir. Dis. 118:617-621 (1978), Gadek, J.E. et al Science 206:1315-1316 (1979) and Beatty, K. et al J. Lab. Clin. Med. 100:186-192 (1982)) and that an "acquired" defect in lung A1Pi is produced in cigarette smokers (Janoff, A. J. Appl. Physiol:Respirat. Environ. Exercise Physical.55:285-293 (1983)).

Consequently, various attempts have been made to develop inhibitors of HLE for potential use against those diseases wherein HLE has been implicated in a pathogenetic role. For example, investigators concerned with the treatment of pulmonary emphysema have tried several methods to improve the inhibitory screen of the lung. One such method is the infusion of pooled A1Pi. This has been effective in replenishing the antiproteinase screen in the lungs of patients with a genetic related A1Pi deficiency, see for example, Gadek, J.E. et al J. Clin. Invest. 68:1158-1165 (1981). However, this is an impractical approach since A1Pi is cleared so rapidly from the circulation that frequent massive infusions are required.

Other investigators are looking into the introduction of inhibitor-producing genetic materials into bacteria by recombinant DNA technologies. However, owing to the complexities of these technologies, progress in this area has been slow. To date no clinically effective elastase inhibitors have been reported. Furthermore, toxicity and antigenicity problems may be presented by materials produced by DNA

technologies. Toxicity and antigenicity also have been the major problems associated with the use of those synthetic elastase inhibitors thus far produced. For example, certain peptide chloromethyl ketones (Powers, J.C. Am. Rev. Respir. Dis. 127:54-58 (1983)) have been shown to be effective *in vivo* (Janoff, A. and Dearing, R. Am. Rev. Respir. Dis. 121:1025-1029 (1980)), Kleinerman, J. et al Am. Rev. Respir. Dis. 121:381-387 (1980) and Ip, M.P.C. et al Am. Rev. Respir. Dis. 124:714-717 (1981)), but these agents have shown nephrotoxicity (Ranga, V. et al Am. Rev. Respir. Dis. 124:613-618 (1981)). More recently, mechanism-based irreversible inhibitors of HLE have been described, e.g., 3-chloroisocoumarin and 3,3-dichlorophthalide (Harper, J.W. et al J. Am. Chem. Soc. 105:6518-6520 (1983)). But here again, nephrotoxity is an issue.

Scientists following another investigatory path have found that HLE can be inhibited by secretory products of certain fungi. These products include "elasnin", a highly alkylated 4-hydroxy-alpha-pyrone from *Streptomyces noboritoensis* (Nakagawa, A. et al J. Org. Chem. A5:3268-3274 (1980)) and "elastatinal", a small peptidyl aldehyde from a different streptomyces strain (Umezawa, H., Aoyagi, T., Okura, A., Morishima, H., Takeuchi, T. and Okawi, Y. J. Antibiot. 26:787-789 (1973) and Okura, A. et al J. Antibiot. 28:337-339 (1975)).

However, few efforts have been made to design substances based on structural knowledge of the reactive sites of naturally-occurring, endogenous elastase inhibitors even though the complete sequence of AlPi is now known (Carrell, R.W. et al Nature 298:329-334 (1982)). In the course of investigating the structure of the reactive sites of naturally-occurring, endogenous elastase inhibitors,

applicants have discovered a new class of naturally-occurring HLE inhibitors. They are found in pyogenic bacteria and can be purified by certain novel processes hereinafter described.

DISCLOSURE OF THE INVENTION

Applicants have discovered a novel class of HLE-inhibitors in pyogenic bacteria, e.g. Streptococcus pneumoniae, Staphylococcus aureus and Klebsiella pneumoniae (pneumococci). It should be noted that, for the purposes of this patent disclosure, the singular term "HLE inhibitor" and the plural term HLE inhibitors should be understood as being interchangeable since more than one HLE inhibitor may exist within the class. It should also be understood that, of these pyogenic bacteria, the pneumococcal inhibitors are the easiest to obtain in large yields. Therefore, they are the ones most extensively characterized. However, as will be indicated by some of the following portions of this patent disclosure, we have reason to believe they are prototypical of this newly discovered class of HLE-inhibitors. Their characterization can begin by noting that they are non-secretory, large molecular weight proteins. They are clearly different from both elasnin and elastatinal in their physical properties and kinetics of inhibition, as well as in their species of origin. Perhaps the most important single attribute of these inhibitors is the fact that they are specific to HLE. This is not true of any other known naturally occurring eukaryotic elastase inhibitor such as, for example, A1Pi or bronchial mucous proteinase inhibitor. Furthermore, since they are derived from bacteria which frequently live in the human body (e.g., pneumococci in nasopharynx, staphylococci on skin), often with no ill effects, they are less likely to be toxic or antigenic than those HLE inhibitors (1) designed on the basis of the known peptide-bond specifications of HLE such as the peptide chloromethylketones previously noted, (2)

derived from genetically engineered bacteria, or (3) discovered through large-scale fungi screening programs.

The HLE inhibitors herein disclosed can be purified according to known procedures; however they can be more effectively purified by a novel affinity column procedure hereinafter described. The first step in this novel process for purifying the HLE inhibitor materials is to bind elastin to an affinity column support material such as, for example, sepharose. Human leukocyte elastase is then bound to the elastin. Since this binding does not require the HLE molecules' active site, the bound HLE molecules have their active sites available for binding the HLE inhibitor molecules which are then introduced into the column as lysates of the pyogenic bacteria. After using a first elution medium to remove contaminants, the bindings between the HLE and the HLE inhibitor molecules are broken by use of a second elution medium having a raised salt concentration, i.e., a salt concentration higher than that of the first elution medium. This breaking of the HLE/HLE inhibitor binding takes place without breaking the binding between the elastase and the elastin, and thereby provides a novel and efficient process for purifying the sought after HLE inhibitor.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the inhibition of human neutrophil elastase by extracts of S. pneumoniae (III), after prolonged preincubation of enzyme with the bacterial protein at 37°C. The extract was prepared by lysis of the bacteria in sodium deoxycholate, centrifugation and extensive dialysis of the supernate. Preincubation was carried out in o.01M Tris at pH 8.0, plus 0.15M NaCl and enzyme assays were carried out in 0.2M Tris buffer, pH 8.0, containing 0.15M NaCl. Sodium deoxycholate alone had no effect on enzyme activity. The (□) line indicates a 30 minute preincubation; the (◇) line indicates a 60 minute preincubation; and the (○) line shows the results of a 90 minute preincubation. The (▲) line indicates a 60 minute preincubation at 0°C.

Figure 2 shows the chromatography at 4°C of S. pneumoniae (type III) extract against neutrophil elastase covalently bound to Sepharose. The running buffer was 0.01 M Tris at pH 8.0. Column dimensions were 1.0 x 10 cm. The solid line (——) indicates absorbance at 280 nm (A280 nm); the shorter dash line (--) indicates inhibitory activity vs neutrophil elastase. Clearly, two separate inhibitors of HLE are present in pneumococcal extracts (see I and II in figure 2). Subsequent experiments revealed that one of the agents was low molecular weight and was inactive in the presence of 1.15M NaCl while the other agent was high molecular weight and retained its activity in 0.15M NaCl.

Figure 3 shows a Lineweaver-Burk analysis (plots of reaction velocity$^{-1}$ vs substrate concentration$^{-1}$) of salt-sensitive and salt-resistant elastase inhibitors derived from pneumococci. Each assay mixture contained 36 ug/ml of

pure human neutrophil elastase and 135 ug/ml of purified salt-sensitive inhibitor or of whole pneumococcal extract (crude salt-resistant inhibitor). The (○) line indicates enzyme alone; the (▲) line indicates enzyme plus salt-sensitive inhibitor purified from type III pneumococci by preparative polyacrylamide gel electrophoresis; the (□) line indicates enzyme plus crude salt-resistant inhibitor from type II pneumococci; and the (●) line indicates enzyme plus crude salt-resistant inhibitor from type III pneumococci. The reaction velocity V, indicates moles of substrate hydrolyzed per minute. The substrate concentration S is given in moles/liter. The graph indicates that the salt-sensitive agent inhibited competitively; the y-intercept (maximal velocity) is identical to that of the enzyme alone, whereas the x-intercept, affinity for substrate of (Km), is different from enzyme alone. By contrast, the salt-resistant agents from types II and III pneumococci inhibited non-competitively as can be observed from the different y-intercept and similar x-intercept values.

Figure 4 shows the chromatography of pneumococcal extract on Sephacryl S-300. The solid line (———) indicates protein concentration expressed as absorbance at 280 nm. The short dashed line (--) indicates percentage inhibition of human neutrophil elastase in the presence of 0.15 NaCl. The long dash line (———) indicates percentage inhibition of human neutrophil elastase in the absence of 0.15M NaCl.

Figure 5 shows the results of SDS-gradient polyacrylamide gel electrophoresis of crude pneumococcal proteins and peak I protein obtained by molecular-sieve chromatography (as in Figure 4). Peak I of Figure 4, which was enriched in specific activity of the salt-resistant inhibitor, also showed

enrichment of one major band of high molecular weight protein, but contained trace amounts of lower molecular weight species as well (see Figure 5, panel A, lane 2). Identification of the enriched high MW band in Figure 5 (panel A, lane 2) as the high molecular-weight, salt-sensitive inhibitor was further supported by the observation that complexes of radio-iodinated neutrophil elastase and this inhibitor eluted in the high MW fraction during molecular-sieving through Sephacryl S-300 (See Figure 6). The molecular weight of this major band (putative salt-resistant inhibitor) was calculated to be 140,000 daltons, cf. arrow vs molecular weight markers in kilodaltons in Figure 5, panel B.

Separate experiments revealed that this inhibitor was not active against trypsin (data not shown) or pancreatic elastase (see Table I). Therefore, a trypsin digestion experiment was performed to confirm that the inhibitor was at least partly protein in nature. Incubation of the partly purified inhibitor with TPCK-trypsin (defined in the Materials and Methods-Proteolytic Enzymes portions of this patent disclosure) for 24 hours at ambient temperature resulted in loss of the major protein band and the appearance of smaller molecular weight fragments (see Figure 5, panel A, lane 3). After incubation with 1mM dithiothreitol and 3M urea, the trypsin-cleavage products were further dissociated into even lower molecular weight fragments. From these results, we have reason to believe that the high molecular weight inhibitor is susceptible to proteolysis and also that the molecule probably contains subunits which are stabilized by hydrophobic associations and/or disulfide bonds. Of considerable interest is the fact that the trypsin-cleavage products retained inhibitory activity against neutrophil elastase.

Figure 6 shows the Sephacryl S-300 chromatography of mixtures of isotope-labelled human neutrophil elastase and pneumococcal extract (type III pneumococcus, reaction in 0.15 M NaCl). Curve 1, i.e., the (— — —) curve indicates $^{14}C$-peptide chloromethyl ketone-treated elastase (100% inactivated) plus bacterial extract. Curve 2 (— —) is as above except treated elastase is 56% inactivated. Curve 3 (———) is as above except that the elastase is 10% inactivated. Elution volumes of $^{125}I$-labelled free elastase and $^{125}I$-labelled elastase complexed with the salt-resistant inhibitor are also marked in this Figure. $V_o$ indicates the Void Volume. Further explanation and interpretation are given in later portions of the patent specifications.

Figure 7 indicates the double-immunodiffusion patterns given by Sephacryl S-300 eluted fractions containing neutrophil elastase complexed with pneumococcal inhibitor. The antiserum to neutrophil elastase is indicated in the central trough; the pure enzyme alone is indicated in the two wells labelled e; all other wells contained selected fractions from curve #3 of Figure 6 (every fifth fraction is numbered in the figure). Wells marked by "X" contained no sample. Gels were eluted to wash out unprecipitated protein, dried and stained with Coomassie blue.

Figure 8 plots the percent activity remaining (100 percent = fractional activity of 1) vs. molar ratio of inhibitor/neutrophil elastase in reaction mixtures. Pure neutrophil elastase (Mr = 32,000) was incubated with partly-purified inhibitor (Sephacryl peak I), and the estimated Mr (i.e., molecular weight as determined by molecular radius) of the inhibitor was taken as about 140,000 (see the "Results" portion of the patent disclosure for further relevant discussions). Extrapolation of the plot to zero percent remaining

activity allowed estimation of the functional molar ratio of inhibitor/enzyme in the complex.

Figure 9 depicts a proposed lysis mechanism of pneumococci cell wall material.

Figure 10 shows chromatographs of pneumococcal lysate on Sepharose 2B and Sephadex G-100.

Figure 11 is a gel-electrophoregram of smaller fragments of the inhibitor.

Figure 12 shows the histologic appearance of mouse lung treated with various mixtures. Panel A represents a buffer control; panels B and C represent 50 ug neutrophil granule extract incubated for 10 minutes at 37°C with 200 ug nonspecific goat immunoglobulin (protein control) and panel D represents 50 ug granule extract incubated as above with 200 ug pneumococcal inhibitor-peptides. Significant hemorrhage can be noted in panels B and C, and a decrease in this manifestation of acute lung injury can be noted in panel D. Goat immunoglobulins alone did not produce acute injury.

## BEST MODES FOR CARRYING OUT THE INVENTION

### BACTERIA SOURCES AND INITIAL EMBODIMENTS

Streptococcus pneumoniae type I (ATCC 9163), type II (ATCC 11733), type III (ATCC 10813) and Staphylococcus aureas (ATCC 25923) were from the American Type Culture Collection (Rockville, Md). Other Streptococcus pneumoniae were obtained from blood cultures of patients with active pneumococcal pneumonia. The Klebsiella pneumoniae was isolated from clinical sources. Representative bacteria were grown in Todd-Hewitt (beef heart-infusion) broth at 37°C for 16-18 hours. Cells were then collected by centrifugation at 1500 x g for 10 minutes, washed three times in cold phosphate-buffered saline, suspended in this same medium and lysed by various known techniques which were sometimes found to have certain specific advantages with regard to the kinds of cells being lysed. For example, sonication in the cold (Sonifer Cell Disruptor, Model W140, Ultrasonics, Inc., Plainview, NY), or incubation for 1 hour at 37°C in the presence of 0.4 percent sodium deoxycholate were often used as the lysis technique. However, the latter procedure was employed only for S. pneumoniae, which shows unique susceptibility to lysis in ionic detergents including bile salts. Another specific adaptation was found in the use of a French press (12,000 pounds per square inch) which easily lyses the Streptococcus pneumoniae but cannot lyse the Staphylococcus aureus. Alumina grinding worked well on Staphylococcus aureus but a slimy capsular material was formed when this technique was used on Streptococcus pneumoniae. Freeze thaw techniques worked well on the Streptococcus pneumoniae but not on

the <u>Staphylococcus aureus</u>. In any event, following lysis, cell debris was removed by high-speed centrifugation. Sodium deoxycholate supernates were extensively dialysed against phosphate-buffered saline, and the extracts were frozen at about -85°C until used.

## PREPARATION OF PROTEOLYTIC ENZYME

The bovine trypsin utilized (freed of contaminating chymotrypsin activity by treatment with tosyl-phenylalanyl-chloromethyl ketone (TPCK)) was that supplied by Worthington Biochemical Corp. (Freehold, NJ). Porcine pancreatic elastase was purchased from Elastin Products Co., Inc. (Pacific, MO). Pure human neutrophil elastase and crude extracts of human neutrophil granules were prepared according to the methods of Feinstein and Janoff:A rapid method of purification of human granulocyte cationic neutral proteases:purification and further characterization of human granulocyte elastase. <u>Biochem</u>. <u>Biophys</u>. <u>Acta</u> 403:493-505 (1975).

## INHIBITION ASSAYS

Inhibition of proteases by crude bacterial extracts or by purified bacterial inhibitors was expressed as a percent of control enzyme activity (without inhibitor) and normalized to the protein content of the extract or inhibitor fraction tested. Elastase activity (neutrophil and pancreatic enzymes) was measured using succinyl-L-alanyl-L-alanyl-L-alanyl -p-nitroanilide (Sigma Chemical Co., St. Louis, MO) as substrate, according to the method of Bieth, Speiss and Wermurth: The synthesis and analytical use of a highly sensitive and convenient substrate of elastase

Biochem. Med. 11:350-357 (1974). Trypsin was assayed using N-benzoyl-D, L-arginine-p-nitroanilide (Sigma Chem. Co.) as the substrate. All enzyme activities were monitored kinetically in a Gilford Model 2400-S Recording Spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH), and rates of hydrolysis were calculated from the linear portions of the curves during the initial 5 to 10 minutes of incubation (22°C). Controls included substrates alone (substrate blanks), substrates plus enzymes without inhibitors (enzyme controls), and substrates plus inhibitors without enzymes (inhibitor blanks). Sodium deoxycholate (0.4%) was demonstrated to be without effect on neutrophil elastase.

Another test procedure was carried out as follows:

Organisms: Streptococcus pneumoniae (Strep PN) was obtained from blood cultures of patients with active pneumococcal pneumonia from the University of Colorado Health Sciences Center, St. Joseph's Hospital, and the Denver V.A. Medical Center. The staphylococcus aureus utilized was a stock reference organism (ATCC #25923). All organisms were stored frozen at -70°C in trypticase soy broth made 1% in dextrose.

Reagents: Tritiated bovine neck ligament elastin was prepared in one of our laboratories. Human neutrophil elastase and crude granule extracts were prepared from various normal subjects and from HL-60 cells (a continuous human cell line derived from a patient with a leukemia of neutrophil-like cells). Porcine pancreatic elastase was purchased from Elastin Products Co. (No. EC134).

Bacterial Growth: Bacteria were grown in trypticase soy broth with 1% dextrose. The broth was innoculated and cultured at 37°C.

Lysate preparation: The broth was removed from an incubator after the culture reached its last third of exponential growth curve as determined by optical density at 540nm and placed in a cold room to arrest growth. Broth was then centrifuged in cold at 20,000 x G for 10 minutes. The pellet was washed and centrifuged again.

KNOWN PROCEDURE FOR INHIBITOR SPECIFICATION

In some experiments, crude bacterial extracts were passed through a column of Sepharose to which pure human neutrophil elastase had first been covalently-linked. The latter was accomplished using cyanogen-bromide activated Sepharose according to the method of Cuatrecasas, P.:Protein purification by affinity chromatography. Derivations of agarose and polyacrylamide beads. J. Biol. Chem. 245:3059-3065 (1970). Chromatography was carried out in 0.01M Tris-HCl buffer (pH 8.0), and individual fractions were monitored for protein absorbance at 280nm and for inhibitory activity against neutrophil elastase.

In other examinations, pneumococcal extracts were chromatographed on Sephacryl S-300, superfine, (Pharmacia Fine Chemicals, Uppsala, Sweden). The fractionation range (Mr) of this gel is $1x10^4$ - $1.5x10^6$ for globular proteins. Column dimensions were 3 cm x 75 cm and the buffer was 0.01M Tris-HCl, pH 8.0, containing 0.15M NaCl. Fractions were collected at a flow rate of 11 ml/hr.

NOVEL PROCEDURE FOR INHIBITOR ENRICHMENT

A novel method by which the initial material used for purification can be enriched in the inhibitor is to suspend the bacteria from which this inhibitor is

to be purified in a nonionic, hypertonic medium such as about 0.6M sucrose. The bacterium are then subjected to lysozyme digestion. Treatment in this manner allows for solubilization of the cell wall constituents (of which this inhibitor appears to be a part) without disrupting the cell membranes. As a result, intracellular substances and cell membranes are not released into the solution and can be spun down leaving the inhibitor in the supernate. Extraneous proteins can then be segregated away, for example, by passing the supernate over a Sepharose 2B chromatography column (see for example Figure 5, column B) or further purified as described below.

## NOVEL PROCEDURE FOR INHIBITOR PURIFICATION

Increased HLE inhibitor purification can be obtained by a novel affinity column technique hereinafter described. This technique can be used as an adjunct to the novel lysozyme digestion procedure described above or it can be used on lysates of the pyogenic bacteria which have not undergone lysozyme digestion. The novel affinity column procedure begins with binding elastin to an insoluble solid support material such as, for example, sepharose. This binding can be accomplished by covalent binding, such as, for example, that achieved by use of a covalent ligand such as cyanogen bromide. HLE is then bound to the elastin by their normal interaction so that at least a portion of the active sites (enzymatic cleavage sites) of the HLE are still available for binding HLE inhibitors. Lysates of the pyogenic bacteria are then introduced to the affinity column so that the HLE inhibitors in the lysates of the pyogenic bacteria become bound to the active site of the neutrophil elastase. Preferably the lysates carrying

the HLE inhibitor are introduced until the elastase molecules become saturated with inhibitor. The affinity column is then eluted with a first elution medium capable of separating contaminating substances from the HLE inhibitors. A low ionic strength neutral buffer (e.g., 0.1M Tris at pH 7.2) can be used for this purpose. Thereafter, the affinity column is eluted with a second elution medium capable of separating the HLE inhibitors from the HLE. A buffer (e.g., a salt medium such as a NaCl medium having a salt concentration of from about 0.01 to about 1 molar) having an ionic strength higher than that of the first elution medium can be used to break the binding between the HLE and the HLE inhibitor without breaking the binding between the HLE and the elastin and thereby providing an efficient method for purifying the HLE inhibitor.

## POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide disc-gel electrophoresis was carried out at pH 8.6 in 12 percent acrylamide separation gels for two and one half hours at 3 mA per gel. Analytical gels were stained with Coomassie blue; preparative gels were manually sliced and selected slices were extracted into 0.01M Tris-Cl buffer, pH 8.0, for 24hr at 37°.

Gradient, sodium-dodecyl-sulfate (SDS) polyacrylamide slab-gel electrophoresis was carried out according to the method of Laemmli:Cleavage of structural proteins during the assembly of the head of bacteriophage $T_4$, Nature 227:680-685 (1970). Various purified proteins were used to calibrate the gels for estimation of molecular weight. After fixation, proteins were visualized by silver-staining as described by Wray, W. et al, Silver staining of

proteins in polyacrylamide gels Anal. Biochem. 118:197-203 (1981).

LABELLING PROCEDURE

Radioactive labelling of pure neutrophil elastase was carried out according to the method of McFarlane A.S.:In vivo behavior of I131-fibrogen, J. Clin. Inves. 42:356-361 (1963), except that the concentration of unlabelled iodine was increased 10-fold. The catalytic site of the enzyme was protected during iodination by addition of 22mM N-succinyl-(L-alanyl)$_3$-p-nitroanilide to the reaction mixture. Under these conditions, catalytic activity of neutrophil elastase was unaffected by the labelling procedure. The $^{125}I$ (as sodium iodide) was from Amersham (Arlington Heights, IL) and had a specific activity of 1.99 mCi/n mole. The final, labelled enzyme had a specific activity of 0.81 mCi/n mole.

ENZYME INACTIVATION

In selected experiments, neutrophil elastase was incubated under various conditions (see "Results" portion of this patent disclosure with $^{14}C$-labelled-N-acetyl-L-alanyl-L-alanyl-L-prolyl-L-valyl chloromethyl ketone (specific activity=2.78 mCi/m mole) to either partially or completely inactivate the enzyme. The preparation was then dialyzed extensively to remove any unreacted peptide chloromethyl ketone, and the inactivated enzyme mixed with pneumococcal extract and analysed for complex-formation.

## PROTEIN ASSAY

Protein concentration of bacterial extracts were measured using the method of Lowry, i.e., Lowry, O.H., N.J. Roseborough, A.L. Farr and R.J. Randall:Protein measurement with the folin phenol reagent J. Biol. Chem. 193:265-275 (1951). Bovine serum albumin was used as the reference.

## IMMUNODIFFUSION TESTS

Neutrophil elastase alone, or Sephacryl S-300 fractions containing complexes of elastase and pneumococcal inhibitor were tested for immunoreactivity against a monospecific rabbit anti-human neutrophil elastase antiserum by double-immunodiffusion in agarose gels. Gels were then washed free of soluble proteins, dried and stained with Coomassie blue to enhance visualization of precipitin lines. The rabbit antiserum was prepared by the methods described by Feinstein and Janoff in Biochem. Biophys. Acta 403:493-505 (1975).

## RESULTS

Figure 1 shows that a second inhibitor of neutrophil elastase could, in fact, be detected in pneumococcal extracts, when the latter were incubated with enzyme in the presence of 0.15 M NaCl for prolonged times at 37°C. Under these conditions, salt-sensitive inhibition was effectively prevented. This inhibition was found both in extracts prepared by sonication as well as by Na deoxycholate-induced lysis. Sodium deoxycholate alone had no inhibitory effect. The NaCl-resistant inhibitory activity could not be attributed to capsular polysaccharides since

the concentration of these in the extracts was low and purified capsular polysaccharides also failed to inhibit the enzyme in the presence of NaCl. In addition, "rough" strains of pneumococci (lacking capsules altogether) still show inhibitory activity (data not shown). Inhibition was not decreased when 0.15M NaCl was added to the assay buffer as well as to the preincubation buffer. Less inhibition was evident when preincubation was carried out for 1 hr at 0°C instead of 37°C (see Figure 1).

## MODE OF INHIBITION COMPETITIVE VS. NONCOMPETITIVE BY EACH OF THE PNEUMOCOCCAL AGENTS:

In order to further investigate the mode of inhibition by pneumococcal agents, the NaCl-sensitive (ionic-interaction-dependent) inhibitor was first isolated. Chromatography of crude extract was carried out in weakly-ionizing buffer against neutrophil elastase bound covalently to Sepharose beads, as shown in Figure 2. The NaCl-resistant inhibitor appeared to elute in the run-through fractions (peak I) along with other proteins. This effect could have been caused by delayed complex formation between this inhibitor and immobilized enzyme at cold temperature. By contrast, faster ionic interactions between the NaCl-sensitive agent and bound enzyme caused passage of this inhibitor to be retarded and it was separately recovered peak-II (see Figure 2). The latter fractions were concentrated by oncotic methods (Aquacide II-A, Calbiochem. La Jolla, CA) and subjected to analytical polyacrylamide disc-gel electrophoresis. The salt-sensitive inhibitor behaved as if it were highly acidic, migrating rapidly towards the anode at pH 8.6. Therefore, subsequent large

scale separation of the salt-sensitive inhibitor was accomplished by extraction of extreme anodal slices of preparative polyacrylamide disc gels.

## INHIBITION METHODS

Each of the agents was then analysed for its mode of inhibition of neutrophil elastase by plotting reaction velocity$^{-1}$ against substrate concentration$^{-1}$ in the presence and absence of the agent. The NaCl-sensitive agent (inhibitor-I), isolated by preparative electrophoresis as above, was tested against pure neutrophil elastase in weakly ionizing buffer. To analyse the second inhibitor, whole pneumococcal extract was preincubated with pure neutrophil elastase at 37°C x 1 hour in the presence of 0.15M NaCl. Results are given in Figure 3, where it can be seen that kinetics of inhibition by inhibitor-I corresponded to a competitive mode, consistent with inhibition by ionic interaction. Maximal velocity (y-intercept) at high substrate concentrations was identical to that of enzyme without inhibitor, while affinity of enzyme for substrate (Km) or x-intercept differed significantly in the presence of this inhibitor. By contrast, inhibitor-II (NaCl-resistant) decreased the maximal reaction velocity of the enzyme, even at saturating substrate concentration, but did not affect the affinity of the enzyme for its substrate (Km). This result is consistent with a non-competitive mode of inhibition for the salt-resistant agent, and suggests formation of stable complexes between neutrophil elastase and this inhibitor.

SEPARATION OF THE INHIBITORS BY MOLECULAR-SIEVE CHROMATOGRAPHY:

Figure 4 shows the chromatographic pattern obtained when crude pneumococcal extract (prepared by lysis of *S. pneumoniae III* in deoxycholate) was passed through a column of Sephacryl S-300 as described under the "Methods" section of this patent application. The bulk of the salt-resistant inhibitory activity against neutrophil elastase (inhibitor-II) appeared with protein eluting soon after the void volume (peak I in the figure) and therefore corresponded to material of high molecular weight. By contrast, when the salt-sensitive inhibitor (purified from polyacrylamide gels) was chromatographed on the same Sephacryl S-300 column, a single-leak of inhibitory activity (in weakly ionizing buffer) appeared at the place indicated in the Figure, corresponding to material of much lower molecular weight. Thus, inhibitor-I is probably normally present in peak VI of the Sephacryl S-300 chromatogram. These data show that the two inhibitors are not only functionally disparate with regard to their mode of inhibition (see preceding section), but are also physically different molecules.

The Inhibitor-II was characterized in much more detail because of the greater likelihood that this agent might be capable of acting under physiological conditions (i.e., in the presence of physiological ion concentrations).

FURTHER CHARACTERIZATION OF INHIBITOR-II

Figure 5 shows the results of SDS-gradient polyacrylamide gel electrophoresis of crude pneumococcal extract and of peak I protein obtained by molecular-sieve chromatography (see preceding

section). Peak I, which was enriched in specific activity of inhibitor-II, also showed enrichment of one major band of high molecular weight protein (panel A, lane 2) and contained, in addition, several fainter bands corresponding to lower molecular weight species. The molecular weight of the major band (putative inhibitor) was calculated to be about 140,000 daltons (see Figure 5, panel B).

Separate experiments revealed that inhibitor-II was not active against trypsin or pancreatic elastase. Therefore, a trypsin digestion experiment was performed to confirm that inhibitor-II was proteinaceous in nature. Incubation of the partly-purified inhibitor-II with TPCK-trypsin (defined in the "Materials" section of this patent application) for 24 hours at ambient temperature resulted in disappearance of the major protein band at about 140,000 daltons. Under non-reducing conditions, electrophorectic analysis revealed smaller protein fragments after trypsin treatment (see Figure 5A, lane 3). Under reducing conditions, however, the trypsin-cleavage products were converted into even lower molecular weight peptides. From these results, we conclude that inhibitor-II is susceptible to proteolysis and also that the molecule probably contains several polypeptide chains crossed-linked by disulfide bonds.

## DEMONSTRATION THAT INHIBITOR-II REQUIRES ACTIVE ENZYME TO FORM AN INHIBITORY GROWTH

Many protein proteinase-inhibitors act non-competitively by posing as substrates for their target enzymes and then forming stable complexes with the latter, once peptide bond attack is initiated, see for example, Travis, J. and G.S. Salvesen:Human plasma

proteinase inhibitors, Ann. Rev. Biochem., 52:655-709 (1983). In these cases, an accessible catalytic-site in the enzyme is required for enzyme-inhibitor complex-formation to take place. To test whether inhibitor-II would similarly interact only with native proteinase, we treated human neutrophil elastase with a specific, irreversible, active-site-directed peptide chloromethyl ketone, see, Powers, J.C. et al:Specificity of porcine pancreatic elastase, human leukocyte elastase and cathepsin G. Inhibition with peptide chloromethyl ketones Biochem. Biophys. Acta 4845:156-166 (1977). Conditions were varied to achieve different degrees of enzyme inactivation and then a determination was made of the extent of complex-formation of the treated enzyme with pneumococcal inhibitor-II. Variable degrees of inactivation were achieved by employing high concentrations of n-succinyl-trialanyl-p-nitroanilide (a low molecular weight substrate of elastase) to compete with the chloromethylketone inactivator for the enzyme's active-site. By varying reaction time as well as the concentration ratio of inactivator/substrate, levels of inactivation ranging from 10%-100% in three separate experiments were achieved.

The peptide chloromethylketone was labelled with $^{14}C$ allowing enzyme which contained bound-inactivator to be detected during chromatographic separation of complexes and free enzyme on Sephacryl S-300. Molecules of enzyme whose catalytic-sites had been protected by substrate and which had therefore remained active were, nevertheless, also labelled with the $^{14}C$-chloromethylketone. This could have occurred by virtue of nonspecific alkylation reactions between the highly reactive chloromethyl ketone and chemical

groups at a distance from the enzyme's active-site (for example, the alpha amino group or tyrosine residues). These mixtures of active, $^{14}C$-labelled, neutrophil elastase and inactive, $^{14}C$-labelled enzyme (dialysed free of unbound inactivator, substrate, and p-nitroaniline were reacted with pneumococcal extract (in the presence of 0.15M NaCl and complexes of enzyme with pneumococcal inhibitor-II were then resolved by Sephacryl S-300 chromatography.

Results are given in Figure 6 where it can be seen that, as predicted, $^{14}C$-label eluted in two distinct peaks. One comes immediately after the void volume (enzyme-inhibitor complexes) followed by a retarded peak of enzyme alone. Again, as predicted, all the radioactivity eluted in this latter peak when 100% inactivated enzyme (confirmed by assay vs succinyl-trialanyl-p-nitroanilide) was reacted with the bacterial extract. For comparison, the same Sephacryl 300 column was also calibrated with $^{125}I$-labelled active neutrophil elastase (free enzyme marker) and $^{125}I$-labelled, active elastase plus pneumococcal extract (marker of enzyme-inhibitor II complexes). Elution volumes of these markers are also given in Figure 6. These results show that complexation of neutrophil elastase with pneumococcal inhibitor-II depended on an open binding-pocket in the enzyme.

When fractions 60-80 in curve #3 of Figure 6 were subjected to double-immunodiffusion vs monospecific, rabbit antiserum against human neutrophil elastase, the pattern shown in Figure 7 resulted. Since no enzyme activity could be detected in these fractions (only elastase that had been inhibited by complexation with pneumococcal inhibitor-II was present), the development of immunoprecipitin lines showing identity

with those given by free elastase suggested that at least some elastase antigenic sites remained exposed in the enzyme-inhibitor complex.

To test whether the inhibitor binds covalently with serine-195 of HLE, elastase was reacted with $^{3}$H-labelled diisopropyl fluorophosphate prior to incubation with the inhibitor. Under such conditions the elastase inhibitor complex is still formed. Moreover, complexes between active elastase and the inhibitor can be dissociated by mild denaturants, supporting the conclusion that they are not covalently bonded.

## SPECIFICITY AND DISTRIBUTION OF THE TWO INHIBITORS:

Table I shows that both pneumococcal inhibitors were specific for neutrophil elastase and neither inhibited porcine pancreatic elastase (or bovine trypsin). In addition, Table I shows that inhibitory activity against neutrophil elastase was also found in extracts of necrotizing organisms (K. pneumoniae and S. aureas), and that S. pneumoniae types I and II (non-necrotizing) were not more inhibitory than S. pneumoniae type III, which is capable of causing tissue destruction.

TABLE I

Distribution and Specificity of the Bacterial Inhibitors

| Assay Buffer | Source of Extract | Extract Protein (ug) | Test Enzyme | Per Cent Enzyme Activity Remaining |
|---|---|---|---|---|
| | S.pn. I | 32 | NE | 58 |
| | S.pn. II | 17 | NE | 76 |
| Tris (o.2M,pH8) | S.pn. III | 17 | NE | 73 |
| | " | 32 | NE | 62 |
| | " | 40 | NE | 57 |
| | "* | 40 | T | 100 |
| | "* | 75 | PPE | 100 |
| | K.pn. | 40 | NE | 55 |
| | " | 40 | PPE | 58 |
| | S.aureas | 40 | NE | 62 |
| | S.pn. II | 70 | NE | 52 |
| | " | 140 | NE | 29 |
| Tris (o.2M,pH8 plus 0.15M NaCl | S.pn. III | 35 | NE | 14 |
| | " | 70 | NE | 10 |
| | " | 70 | PPE | 100 |
| | " | 140 | PPE | 100 |
| | K.pn. | 70 | NE | 92 |
| | " | 140 | NE | 19 |

NE = neutrophil elastase (2 ug pure enzyme or 20 ug crude neutrophil granule protein)

PPE = porcine pancreatic elastase (0.2 ug crystalline enzyme)

T = bovine trypsin (1.0 ug crystalline enzyme)

S.pn. = Streptococcus pneumoniae

K.pn. = Klebsiella pneumoniae

S. aureus = Staphylococcus aureus

* = Three separate bacterial extracts were tested in these cases.

## CHARACTER OF INHIBITORS

This discovery provides the first characterization of two, different neutrophil elastase inhibitors in pneumococci. Our data shows that inhibition by one of these agents (a low molecular weight, acidic substance that acts competitively and is dependent on ionic interactions to bind with the cationic enzyme) is suppressed by physiological concentration of NaCl. This inhibitor is therefore unlikely to play an important role *in vivo*. The second inhibitor however is, by contrast, active in the presence of 0.15M NaCl. This agent inhibits non-competitively and requires an open binding-pocket but not an active catalytic site in its target enzyme to form inhibitory complexes with the latter. It appears to form a 1:1 molar complex with neutrophil elastase. Both pneumococcal inhibitors are specific for human neutrophil elastase; neither inhibits porcine pancreatic elastase. The second inhibitor's apparent molecular weight is about 140,000 daltons, yet it does not mask antigenic sites of the enzyme upon complexation with it. The high molecular weight inhibitor is sensitive to trypsin, and its tryptic degradation fragments are susceptible to further dissociation by reduction. Treatment of pneumococci with deoxycholic acid probably activates an autolytic enzyme in the cells which strips the peptide cross-links out of the cell wall peptidoglycan by cleaving bonds between alanine and muramic acid residues (see arrows, Fig 9). The pneumococci are lysed in the process, releasing free glycan strands

(large molecular weight), peptide bridges (low molecular weight), a number of large molecular weight membrane-associated proteins and glycoproteins, as well as cytoplasmic constituents.

When this lysate is chromatographed on Sepharose 2B, the first peak after the void-volume contains a predominant large molecular weight protein or glycoprotein of 140,000 daltons which make a membrane protein (MP) similar to the high MW cell-surface glycoprotein antigens of other streptococci (see for example, Dorn, et al.: Extracellular Antigens of Serotype III Group B Streptococci, Infect. Immun. 30:890-893, (1980) and OMP derived from gonococci. As would be expected, peak I of the Sepharose 2B chromatogram is also enriched in glycan fragments released during deoxycholate-induced lysis (Fig 9). However, most of the contaminating glycan can be removed from Sepharose peak I by treatment with lysozyme (see Fig 10, panel E).

Inhibitory activity against human leukocyte elastase is associated with the large molecular weight protein or glycoprotein released during deoxycholate-induced lysis and recovered in Sepharose peak I (see panel A of Fig. 10). This protein (or glycoprotein) inhibitor is susceptible to degradation by beta glucuronidase (Fig. 10 panels B and C) or trypsin (Fig. 10 panel D). In either case, smaller fragments ranging in molecular weight between 22 and 45 kilodaltons can then be recovered from G-100 Sephadex columns (Fig. 10 panels C and D) and can be demonstrated on SDS-gradient polyacrylamide gels (Fig. 11). It is also of the utmost importance that these smaller fragments retain inhibitory activity against neutrophil elastase (Fig. 10 panels C and D). This fact should also facilitate the elucidation of the active site structure of the inhibitor.

To summarize our overall characterization of the inhibitor we first note that it has a high molecular weight (140,000 daltons), but inhibitory activity is retained by smaller tryptic-peptides derived from the native agent. The inhibitor is specific for neutrophil elastase and it acts non-competitively. Complex-formation between neutrophil elastase and the pneumococcal agent does not require covalent interaction of the inhibitor with serine-195 in the enzyme, since elastase inactivated by $^{3}$H-labeled diisopropyl fluorophosphate can still form such complexes. Moreover, complexes between active elastase and pneumococcal inhibitor can be dissociated by mild denaturants. On the other hand, enzyme whose substrate-binding pocket is blocked by a peptide chloromthylketone does not react with pneumococcal inhibitor, suggesting that specific recognition-sites on the inhibitor, may interact non-covalently with the protease.

Finally, with respect to the potential physiological role of the major elastase inhibitor herein described, the following points are worth noting. First, solubilization of the inhibitor by deoxycholic acid-induced disruption of pneumococcal cell walls or by sonication of the bacteria may imply an intracellular localization of the agent and this, in turn, could raise questions regarding its ability to interact with extracellular enzyme. It should be remembered, however, that the same disintegration procedures also release molecules associated with cell wall peptidoglycan (see Figure 9). In this latter location, the inhibitor could have ready access to extracellular elastase. Furthermore, even if the agent were to be intracellular in location, following phagocytosis and lysis of pneumococci by neutrophils (or by antibody and complement in lung secretions),

the inhibitor would be released and could then inactivate elastase. With respect to the rate of this inactivation, there is evidence of a slow association of the pneumococcol with neutrophil elastase (see Figure 1). Effective in vivo inhibition usually depends on fast association rates between inhibitors and their target enzymes. However, we will show in the next section of this patent application that the pneumococcal inhibitor can protect mouse lung in vivo, against acute lung injury produced by neutrophil granule extracts, even when inhibitor and enzyme are administered immediately after mixing.

## IN VIVO PROTECTION PROVIDED BY INHIBITOR

Further studies were conducted to determine if the pneumococcal inhibitor could effectively block the activity of human neutrophil elastase in vivo. If so, this might help account for the minimal lung destruction seen in most pneumococcal pneumonias. Moreover, it seemed important to test this question because non-covalent bonding of the inhibitor to the enzyme might permit the latter to dissociate under in vivo conditions. In this event, the physiological relevance of the inhibitor would be open to question.

A model of acute lung injury was chosen to test the in vivo effectiveness of pneumococcal inhibitor. The sequestration of circulating $^{125}$I-albumin in the lungs, lung wet weight and lung hemorrhage caused by intratracheal protease instillation was measured. Both native pneumococcal inhibitor and its active tryptic peptides were tested for protection against these effects of the protease. Whole neutrophil granule extracts were employed as the source of leukocyte elastase in order to include other neutrophil granule constituents in the model. Because

these are also secreted into pneumonic exudates, along with elastase, it was felt that whole granule extracts would more closely duplicate naturally-occuring events.

## IN VIVO TEST METHODS

Human neutrophil granule extract was prepared according to the method of Feinstein, G. and A. Janoff: A rapid method of purification of human granulocyte cationic neutral proteases:purification and further characterization of human granulocyte elastase Biochem. Biophys. Acta 403:493-505(1975). The pneumococcal inhibitor used was the large molecular weight fraction derived by Sepharose-2B chromatography of bacterial extracts, the latter having been prepared by lysis of washed cells in deoxycholic acid. Gel-electrophoresis data and previously discussed experiments had shown that this fraction contained the active 140,000 dalton elastase-inhibitor. Other trials had shown that this pneumococcal fraction was not toxic to mice (data not shown), nor did it produce acute lung injury by itself (see Table II). In addition to the intact inhibitor, active tryptic-fragments of the pneumococcal agent were also tested. These were prepared by 24 hour incubation of the large molecular weight fraction at room temperature with 2% (wt:wt) TPCK-trypsin, followed by inactivation of trypsin with N-tosyl-lysyl-chloromethyl ketone and extensive dialysis to remove the unbound inactivator.

Male, Swiss-Webster mice weighing between 25 and 30 g were anesthetized with intraperitoneal ketamine (Ketaset, Bristol Laboratories, Syracuse, NY). Each animal then received an intravenous injection of $^{125}$I-labelled bovine serum albumin (dose = 1.3 mg,

specific activity = 0.314 uCi/mg). Immediately thereafter, the mice were treated with mixtures of granule extract and inhibitors (native or tryptic peptides). These mixtures were delivered to the lungs by a transoral technique, as described by Janoff, A. and R. Dearing:Prevention of elastase-induced experimental emphysema by oral administration of a synthetic elastase inhibitor Am. Rev. Respir. Dis. 121:1025-1029 (1980). Control mice received granule extract mixed with TRIS buffer used to dissolve the inhibitor, or inhibitor mixed with PBS buffer used to dissolve granule proteins, or a mixture of the two buffers. Additional control groups were treated with granule extract mixed with goat immunoglobulins to serve as a source of nonspecific protein added at the same concentration as the active pneumococcal agent.

In some experiments, the granule extract and inhibitor were premixed and incubated at 37° for 10 minutes before instillation, while in other trials the reagents were mixed at room temperature and instilled into mice immediately thereafter (0 min groups).

Lungs were removed at time of death of the animal or after 1 hr in survivors, and acute lung injury was assessed using three methods: (a) total absorbance (at 412 nm) of hemoglobin in clarified supernates of 2.5% (wt:vol) lung homogenates prepared by grinding the tissues in distilled water; (b) cpm of $^{125}I$-albumin in the lung, expressed as a percentage of injected dose and normalized either to lung dry weight or to body weight; and (c) lung wet wt/body wt ratios.

Bovine serum albumin (Sigma Chem. Co., St. Louis, MO) was radiolabelled with $^{125}I$ using Bolton-Hunter reagent (New England Nuclear, Boston, MA) according to the manufacturer's directions. Protein determinations were done according to the

method of Lowry with bovine serum albumin as a standard. The statistical significance of difference between means for various experimental and treated groups was determined using Student's T-test.

## IN VIVO TEST RESULTS

Four separate experiments involving over 100 mice were done, and the results are hereinafter summarized in Tables II and III. In all cases, 50 ug of granule extract protein and 200 ug of whole pneumococcal inhibitor protein or tryptic peptides were used. When goat immunoglobulin was used as a control protein, 200 ug were similarly employed. As can be seen from the data in Table II, the native pneumococcal inhibitor gave significant protection against acute lung injury caused by neutrophil granule extract, when the inhibitor and extract were preincubated for 10 minutes at 37°C before instilling the mixture into the mouse lungs. Protection was evident using all three parameters of acute lung injury (lung wet wt, $^{125}$I-albumin sequestration, and hemoglobin absorbance of tissue extracts.

TABLE II

Experiments With Intact Pneumococcal Inhibitor Pre-Incubated With Granule Extract For 10 Minutes

| Treatment (no. mice) | $^{125}$I-albumin (% dose recovered in lung/g body wt) | Lung wet wt (mg)/Body wt (g) | Total calculated absorbance: 2.5% lung homogenate supernate at 412 nm |
|---|---|---|---|
| buffer control (5) | 0.035<br>0.010 | 0.0058<br>0.0003 | nd |
| granule extract alone (9) | 0.066[a]<br>0.022 | 0.0081[a]<br>0.0014 | 29.4<br>0.7 |
| granule extract + inhibitor (11) | 0.045[b,d]<br>0.014 | 0.0066[b]<br>0.0009 | 17.5[c]<br>0.9 |
| inhibitor alone (5) | 0.035[d]<br>0.007 | 0.0059[d]<br>0.0001 | nd |

(a) differs significantly from buffer control ($p < 0.01$)

(b) differs significantly from granule extract alone ($p < 0.05$)

(c) differs significantly from granule extract alone ($p < 0.01$)

(d) not significantly different from buffer control

Table III summarizes the results of experiments using active tryptic peptides of the pneumococcal inhibitor. These were effective whether preincubation was for 10 minutes at 37° or for zero minutes at 22°, although greater protection was observed after longer preincubation intervals. Incubation of neutrophil

granule extract with nonspecific goat immunoglogulins did not alter the ability of granule components to produce lung injury.

TABLE III

Experiments With Tryptic Peptides of Pneumococcal Inhibitor Pre-Incubated With Granule Extracts For 10 Minutes (in Panel A) or 0 minutes (in Panel B)

| Treatment (no) | $^{125}$I-albumin (% dose/ body wt) | $^{125}$I-albumin (% dose/ lung dry wt) | Lung Wet wt/body wt | $A_{412nm}$ |
|---|---|---|---|---|
| (A) | | | | |
| buffer control (8) | 0.030 ± 0.007 | 28.8 ± 5.2 | 0.0068 ± 0.0006 | 15.4 ± 3.2 |
| granule extract (16) | 0.111[a] ± 0.040 | 77.8[a] ± 18.0 | 0.0099[a] ± 0.0019 | 30.7[a] ± 8.1 |
| granule extract + goat globulins (8) | 0.105[a] ± 0.038 | 181.3[a] ± 60.4 | 0.0101[a] ± 0.0017 | 30.7[a] ± 6.8 |
| granule extract + peptides (16) | 0.047[b] ± 0.012 | 40.9[b] ± 9.9 | 0.0080[b] ± 0.0012 | 17.6[b,d] ± 5.5 |
| (B) | | | | |
| buffer control (8) | 0.036 ± 0.019 | 36.8 ± 12.1 | 0.0055 ± 0.0005 | 22.7 ± 4.7 |
| granule extract + goat globulins (8) | 0.077[a] ± 0.021 | 61.0[a] ± 16.0 | 0.0090[a] ± 0.0010 | 37.0[a] ± 4.4 |
| granule extract peptides (8) | 0.055[c] ± 0.018 | 56.2 ± 16.1 | 0.0075[c] ± 0.0014 | 30.0[c] ± 6.6 |

(a) differs significantly from buffer control ($p <$ 0.01)
(b) differs significantly from granule extract and/or granule extract + globulins ($p < 0.001$
(c) differs significantly from granule extract and/or granule extract + globulins ($p < 0.05$)
(d) not significantly different from buffer control

Figure 12 shows the typical histologic appearance of buffer-treated mouse lung (panel A) vs lung tissue from animals receiving granule extract that had either been pre-incubated for 10 minutes at 37°C with goat immunoglobulins as a protein control (Panels B and C), or with inhibitor-peptides (panel D). It is evident that the human neutrophil granule extract produced severe, acute lung injury in the mouse. It was primarily characterized by edema and hemorrhage. These responses were significantly reduced by the pneumococcal agent.

## DISCUSSION OF IN VIVO TEST RESULTS

The experiments herein described show that an inhibitor of neutrophil elastase obtained from pneumococci protected mouse lung against acute tissue injury produced by human neutrophil granule extracts. The degree of protection was related to the length of preincubation of granule extract with pneumococcal agent, and protection was enhanced when lower molecular weight tryptic-peptides, derived from the native inhibitor were used. Protection was not due to nonspecific effects of the added pneumococcal protection since equal amounts of goat immunoglobulins used in place of the pneumococcal inhibitor did not prevent injury.

Although the pneumococcal agent specifically inhibits neutrophil elastase and is inactive against other proteases (trypsin, pancreatic elastase),

unfractionated neutrophil granule extracts were used rather than pure neutrophil elastase in the present study. This seemed appropriate, since neutrophil-mediated acute lung injury may also involve other granule components such as: (1) cathepsin G, see Boudier, C., P. Laurent and J.G. Bieth: Leukoproteinases and pulmonary emphysema:cathepsin G and other chymotrypsin-like proteinases enhance the elastolytic activity of elastase on lung elastin, Adv. Expt. Med. 167:313-317 (1980), (2) collagenase, (see, Lazarus, G. S., J. R. Daniels, R. S. Brown, H. A. Bladen and H. M. Fullmer:Degradation of collagen by a human granulocyte collagenolytic system, J. Clin. Invest. 47:2622-2628 (1968)), and (3) oxidants formed by the action of myeloperoxidases (another granule constituent), (see Clark, R. A.:Toxic effects of myeloperoxidase and $H_2O_2$ secreted by neutrophils exposed to a soluble stimulus, Clin. Res. 27:209A (abstr) (1980); Carp, H. and A. Janoff: Potential mediator of inflammation. Phagocyte-derived oxidants suppress the elastase-inhibitory capacity of alpha 1-proteinase inhibitor *in vitro*, J. Clin. Invest. 66:987-995 (1980) and Greenwald, R. A. and W. W. Moy:Effect of oxygen-derived free radicals on hyaluronic acid. Arthritis Rheum. 23:455-463 (1980)). Under these conditions, the pneumococcal agent still gave significant protection.

Previous chemical studies showed that the pneumococcal agent does not form a covalent complex with the active-site serine residue in neutrophil elastase. However, elastase-inhibitor complexes appear to be stabilized by hydrophobic associations and inhibition is non-competitive. Perhaps for this reason complexes can form *in vivo* even in the presence of lung substrates for neutrophil elastase, and this could explain the partial protection observed when

preparations of granule extract and inhibitory-peptides were instilled immediately after mixing.

Thus, by virtue of their ability to specifically inhibit human leukocyte elastase, the above described inhibitors as well as their active site fragments can be used in treating a wide variety of diseases which produce elastolytic effects as part of their disease processes. Such diseases would include, but not be limited to, pulmonary emphysema, pneumonia (viral, bacterial, fungal, protozoan), abscesses, chronic bronchitis, hyaline membrane disease, adult respiratory distress syndrome (ARDS), vasculitis, glomerulonephritis, bronchiectasis, interstitial lung disease, atherosclerosis, arthritis, psoriasis, cystic fibrosis, pemphigus, fasciitis and cellulitis.

Obviously, treatment of such diseases by these inhibitors can be by any convenient delivery method, e.g., inhalation, subcutaneous, intramuscular or intravenous injection, oral administration, salves, ointments or suppositories. It is also obvious that the inhibitors may be admixed with pharmaceutically acceptable carriers and/or other pharmaceutically compatible medicaments.

Likewise, while the human leukocyte elastase inhibitors, the herein described novel methods for their enrichment and purificaiton as well as the herein described methods for their use have been described in great detail, it will be obvious to those skilled in this art that many modifications and/or extensions of these details can be made without departing from the scope and spirit of the teachings of this patent disclosure. Use of these inhibitors in the treatment of elastolytic diseases in humans would be an example of one obvious extension of these teachings.

WHAT IS CLAIMED IS:

1. Proteinaceous human leukocyte elastase inhibitors derived from pyogenic bacteria and specific to human leukocyte elastase.

2. The proteinaceous human leukocyte elastase inhibitors of claim 1 wherein the pyogenic bacteria are selected from the group consisting of Streptococcus pneumoniae, Klebsiella pneumoniae and Staphylococcus aureus.

3. The proteinaceous human leukocyte elastase inhibitors of claim 1 wherein the pyogenic bacteria are Streptococcus pneumoniae.

4. Proteinaceous human leukocyte elastase inhibitors, derived from pyogenic bacteria, specific to human leukocyte elastase and further characterized as being non-secretory, high molecular weight proteins capable of remaining active in the presence of 0.15 M NaCl, acting in a non-competitive manner with human leukocyte elastase, and consisting of subunits stabilized by hydrophobic associations and/or disulfide bonds.

5. The human leukocyte elastase inhibitors of claim 4 wherein the inhibitors have an apparent molecular weight of about 140,000 daltons.

6. The proteinaceous human leukocyte elastase inhibitors of claim 4 wherein the pyogenic bacteria are pneumococci selected from the group consisting of Streptococcus pneumoniae, Klebsiella pneumoniae and Staphylococcus aureus.

7. The proteinaceous human leukocyte elastase inhibitors of claim 4 wherein the pyogenic bacteria are Streptococcus pneumoniae.

8. Proteinaceous human leukocyte elastase inhibitors which are fragments of human leukocyte elastase inhibitors derived from pyogenic bacteria, said fragments having active sites which are active against human leukocyte elastase and are further characterized as being derived from non-secretory, large molecular weight proteins capable of remaining active in the presence of 0.15 M NaCl, acting in a non-competitive manner with human leukocyte elastase, and consisting of subunits stabilized by hydrophobic associations and/or disulfide bonds.

9. The fragments of claim 8 wherein said fragments have an apparent molecular weight of less than about 140,000 daltons.

10. The proteinaceous human leukocyte elastase inhibitor fragments of claim 8 which are derived from the pyogenic bacteria selected from the group consisting of Streptococcus pneumoniae, Klebsiella pneumoniae and Staphylococcus aureus.

11. The proteinaceous human leukocyte elastase inhibitor fragments of claim 8 which are derived from Streptococcus pneumoniae.

12. A method of treating diseases which have elastolysis as part of their disease processes comprising administering an effective amount of human leukocyte elastase inhibitors which are derived from pyogenic bacteria.

13. The method of claim 12 wherein the human leukocyte elastase inhibitors are derived from pyogenic bacteria selected from the group consisting of Streptococcus pneumoniae, Klebsiella pneumoniae and Staphylococcus aureus.

14. The method of claim 12 wherein the human leukocyte elastase inhibitors are derived from Streptococcus pneumoniae.

15. The method of claim 12 wherein the diseases having elastolysis as part of their disease processes are selected from the group consisting of pulmonary emphysema, pneumonia (viral, bacterial, fungal, protozoan), abscesses, chronic bronchitis, hyaline membrane disease, adult respiratory distress syndrome (ARDS), vasculitis, glomerulonephritis, bronchiectasis, interstitial lung disease, atherosclerosis, arthritis, psoriasis, cystic fibrosis, pemphigus, fasciitis and cellulitis.

16. A method of purifying human leukocyte elastase inhibitors derived from pyogenic bacteria comprising:

(1) providing an affinity column by binding elastin to an insoluble support material;

(2) binding human leukocyte elastase to the elastin so that a portion of the active sites of the human leukocyte elastase are available for binding human leukocyte elastase inhibitors;

(3) introducing lysates of the pyogenic bacteria to the affinity column so that neutrophil elastase inhibitors in the lysates of the pyogenic bacteria become bound to the active site of the neutrophil elastase;

(4) eluting the affinity column with a first elution medium which is capable of separating contaminating substances from the human leukocyte elastase inhibitors; and

(5) eluting the affinity column with a second elution medium which is capable of separating the human leukocyte elastase inhibitors from the human leukocyte elastase.

17. The method of claim 16 wherein the insoluble support material is sepharose.

18. The method of claim 16 wherein the second elution medium has a salt concentration higher than that of the first elution medium.

19. A method of enriching human leukocyte elastase inhibitors derived from pyogenic bacteria comprising:

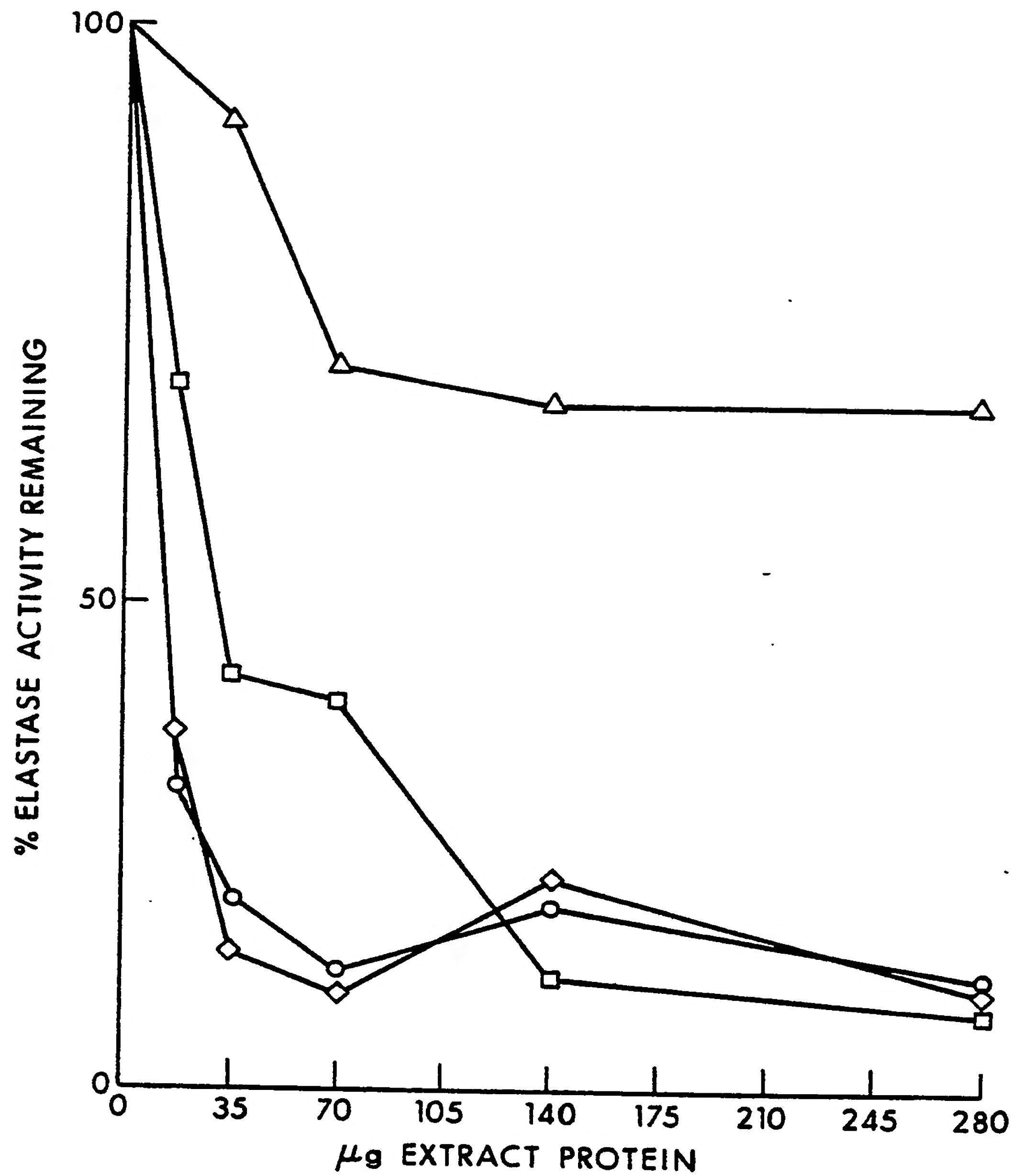
(1) lysozyme digestion of pyogenic bacteria suspended in a nonionic, hypertonic medium such that bacteria cell wall components are solubilized without disrupting bacteria cell membranes and thereby minimizing release of intracellular substances; and

(2) segregating extraneous proteins from the human leukocyte elastase inhibitors.

20. The method of claim 19 wherein the nonionic, hypertonic medium is sucrose having a concentration of about 0.6M.

21. The method of claim 19 wherein the segregating of extraneous proteins from the human leukocyte elastase inhibitor is by chromatography of a cell supernate on Sepharose 2B.

*Fig. 1*


100
50
0
% ELASTASE ACTIVITY REMAINING
0
35
70
105
140
175
210
245
280
μg EXTRACT PROTEIN

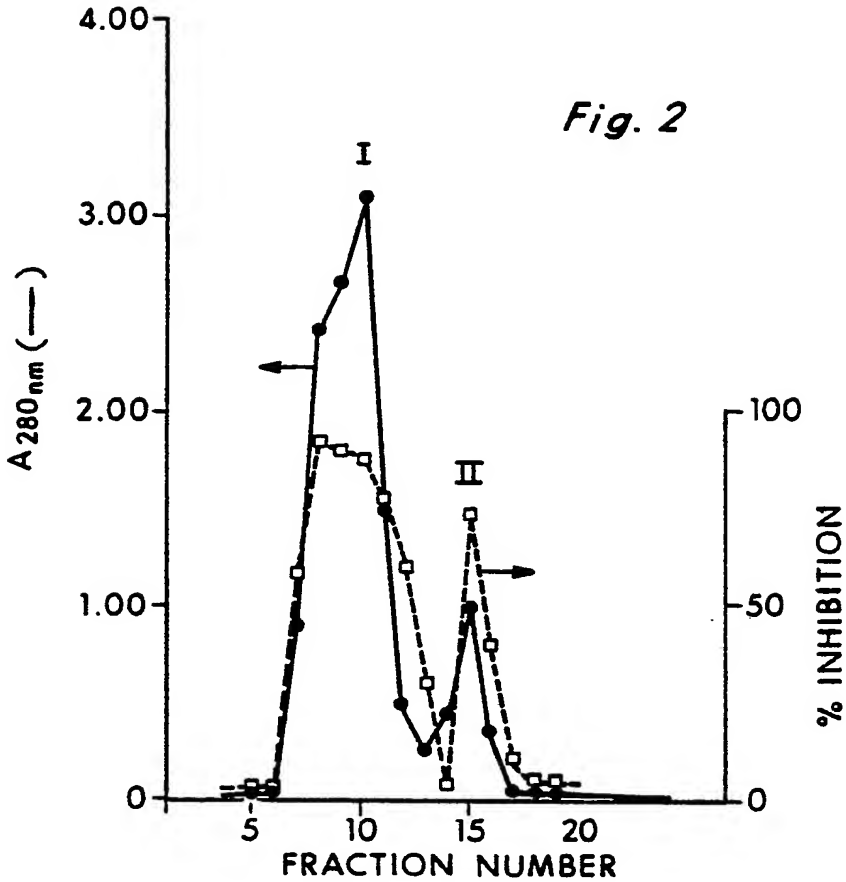
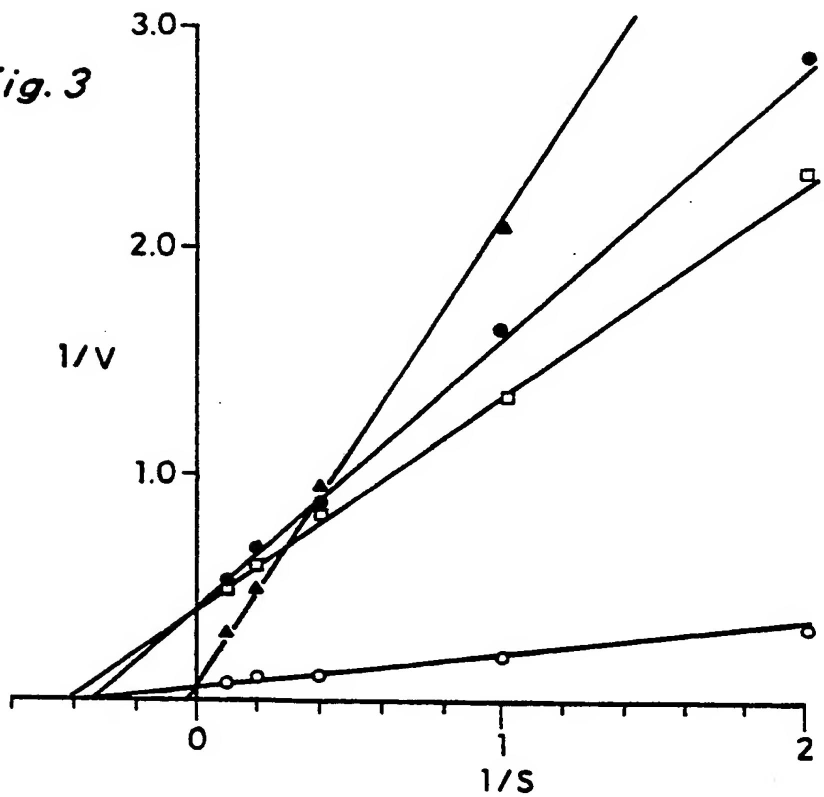



Fig. 2

Fig. 3




SUBSTITUTE SHEET

Fig. 4

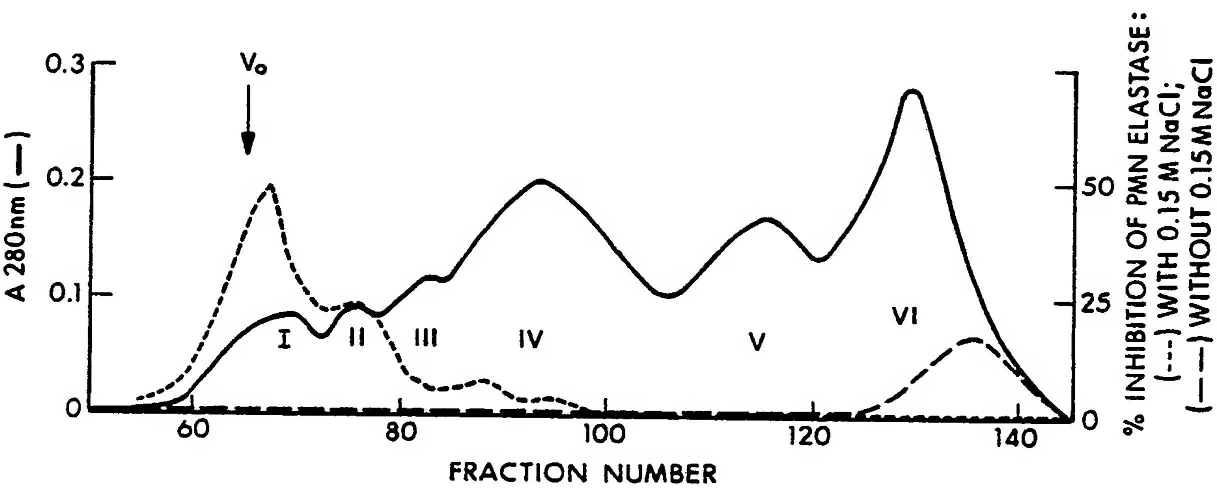

0.3
0.2
0.1
0
A 280nm (—)
Vo
I
II
III
IV
V
VI
60
80
100
120
140
FRACTION NUMBER
50
25
0
% INHIBITION OF PMN ELASTASE:
(---) WITH 0.15 M NaCl;
(— —) WITHOUT 0.15 M NaCl

Fig. 5

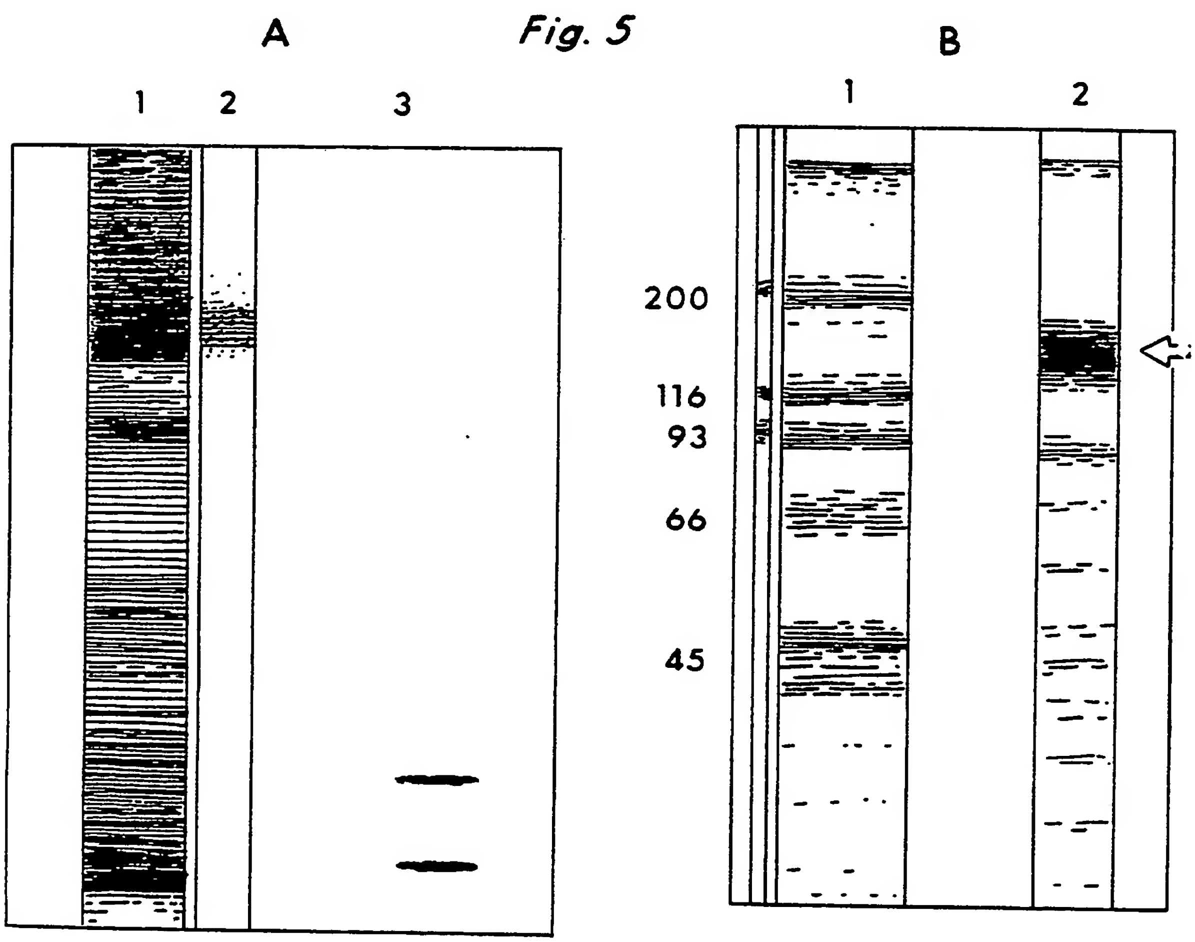

A
1
2
3
B
1
2
200
116
93
66
45

Fig. 6

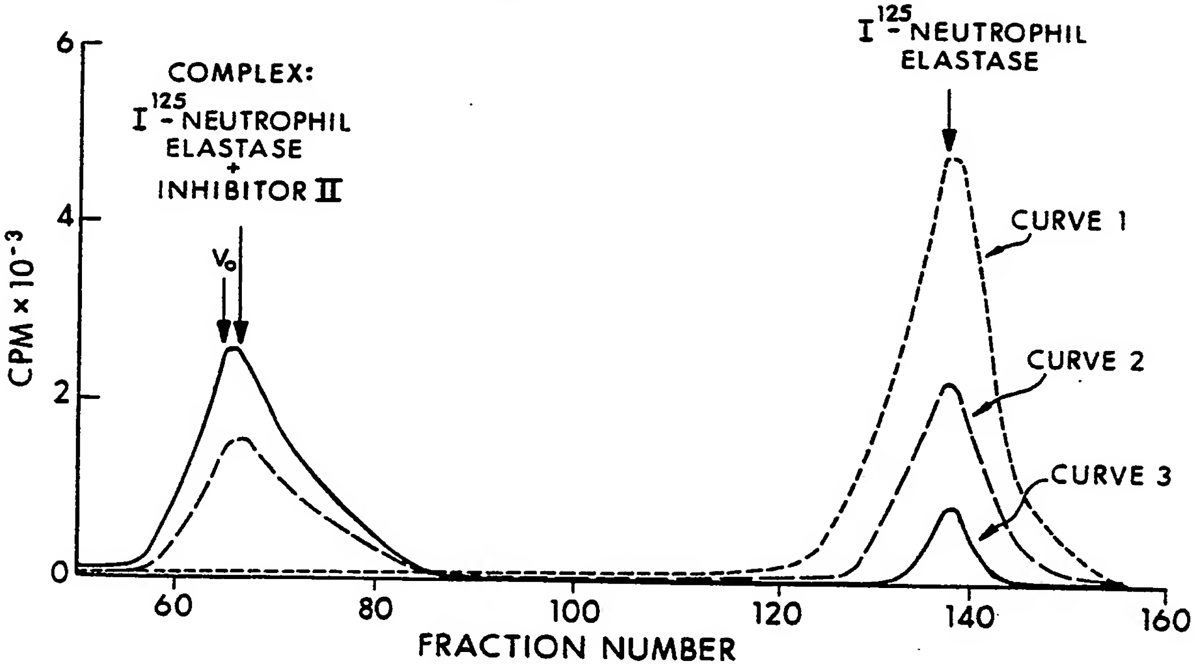

COMPLEX:
I125-NEUTROPHIL ELASTASE + INHIBITOR II
$V_o$
I125-NEUTROPHIL ELASTASE
CURVE 1
CURVE 2
CURVE 3
CPM × 10⁻³
0
2
4
6
60
80
100
120
140
160
FRACTION NUMBER

Fig. 7

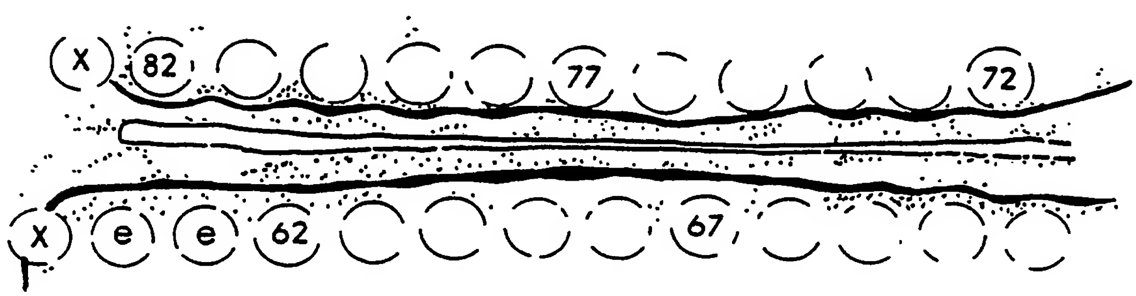

X
82
77
72
X
e
e
62
67

*Fig. 8*

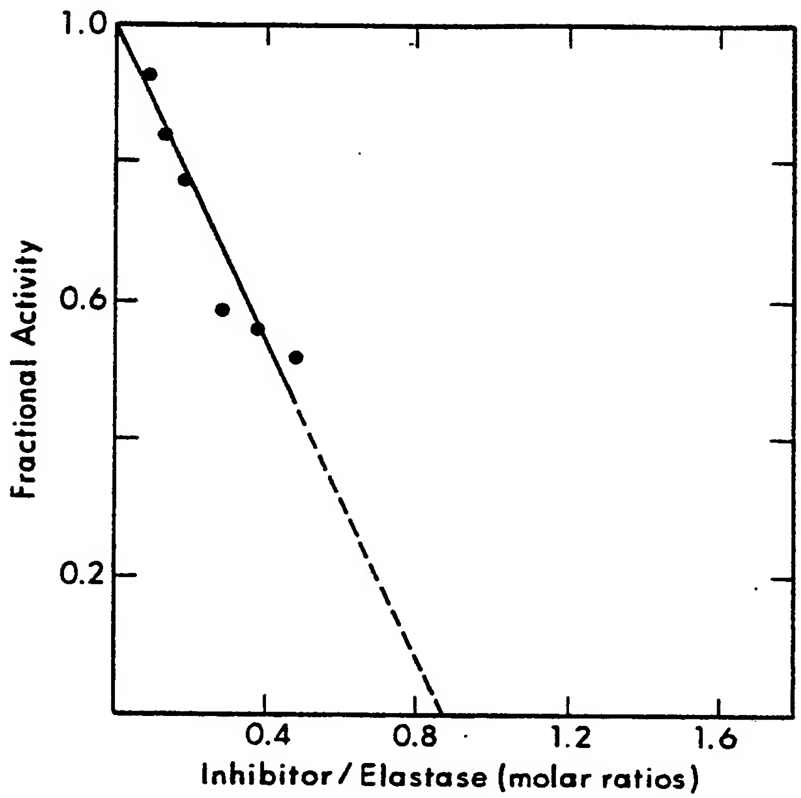
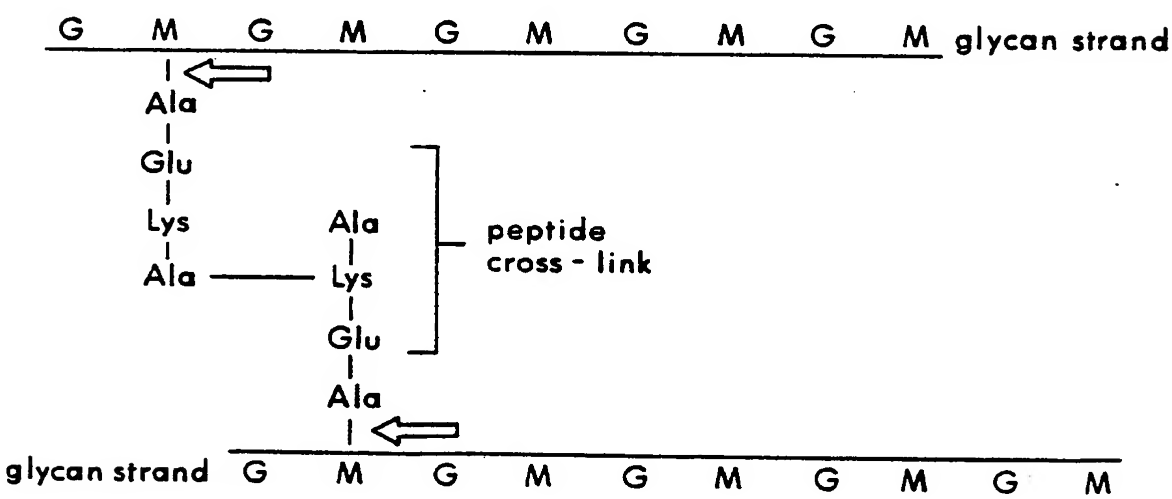



*Fig. 9*

PNEUMOCOCEAL CELL WALL PEPTIDOGLYCAN




G = N - acetyl glucosamin M = N - acetyl muramic acid

⇦ = Site of attack by "autalytic enzyme", which is rel as d by treatment with deoxycholic acid.

*Fig. 10a*

Sepharose 2B chromatogram of crude pneumococcal extract (type III).

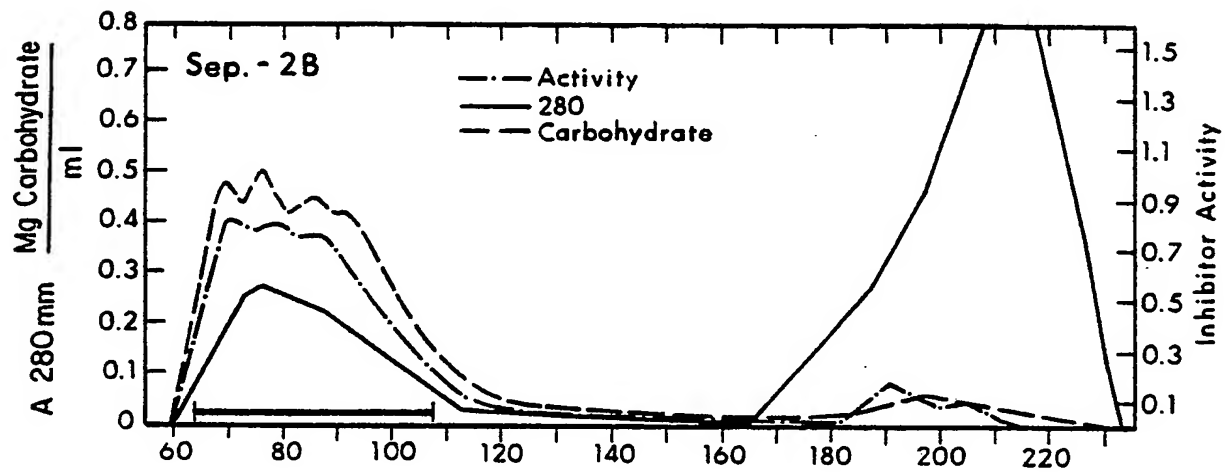



*Fig. 10b*

Sepharose 2B chromatogram after beta glucuronidase digestion.

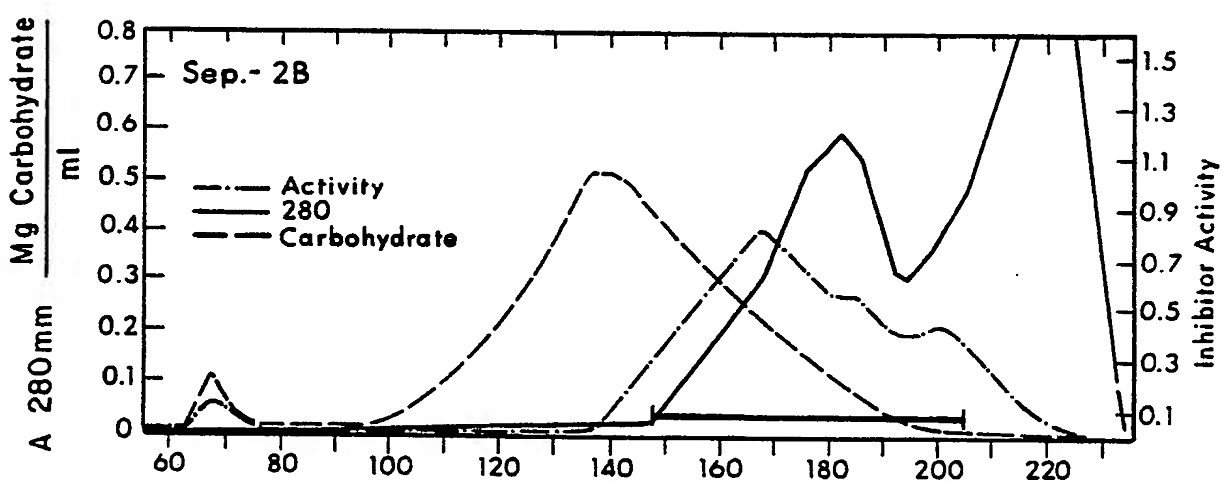


*Fig. 10c*

The active fraction from (B) pooled and re-chromatographed on Sephadex G100.

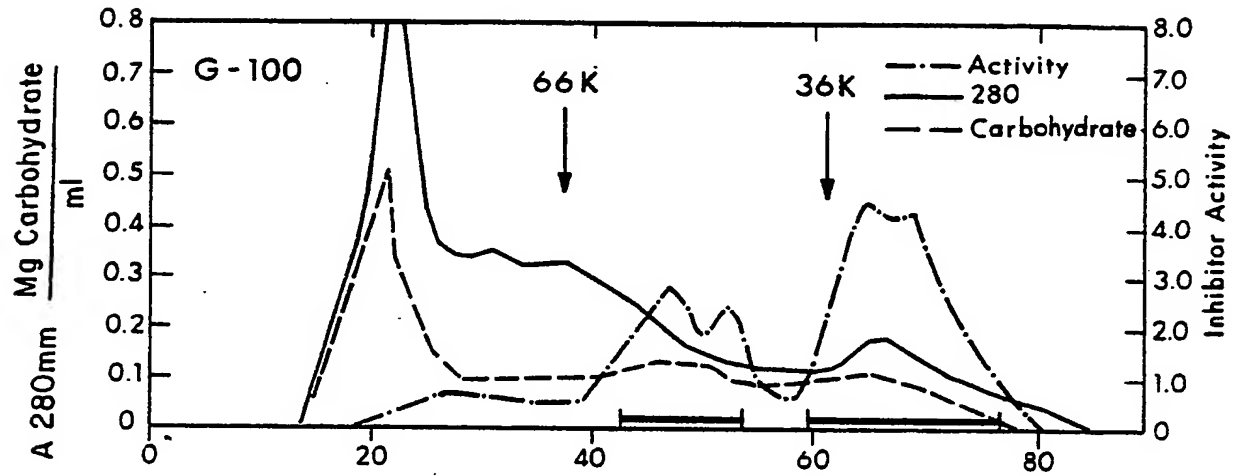



*Fig. 10d*

Sephadex G100 chromatogram after trypsin digestion of peak 1 from panel A.

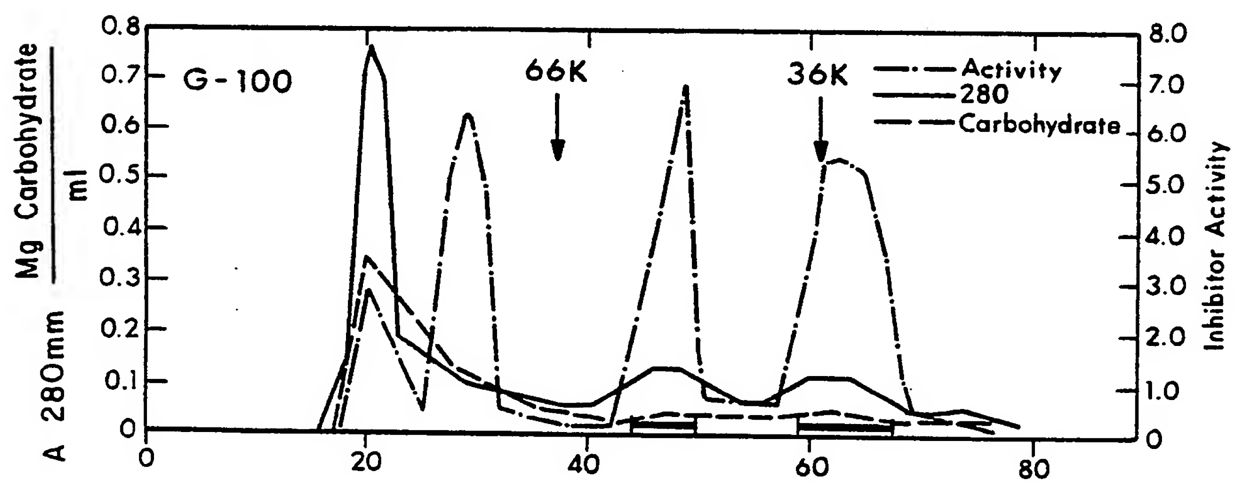


*Fig. 10e*

Sepharose 2B chromatogram after lysozyme treatment of peak 1 from panel A.

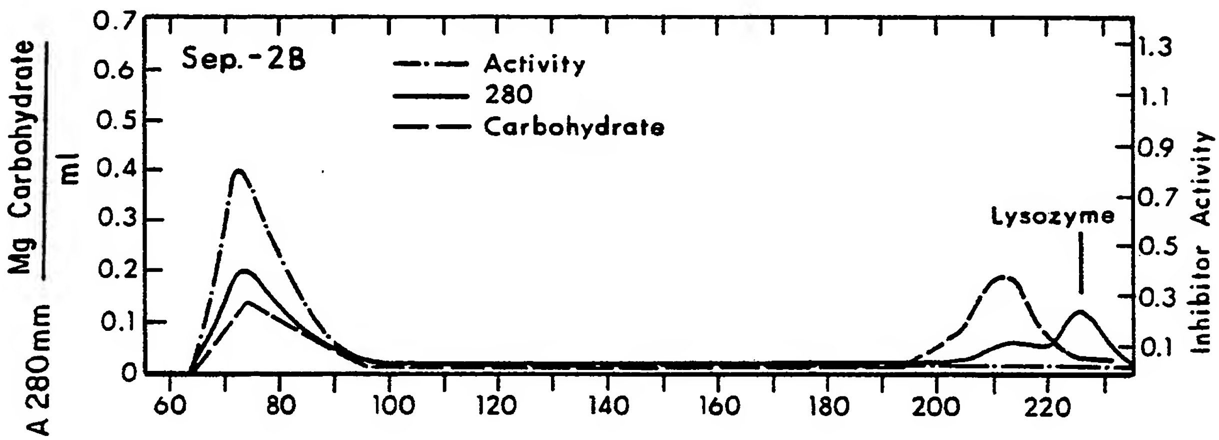
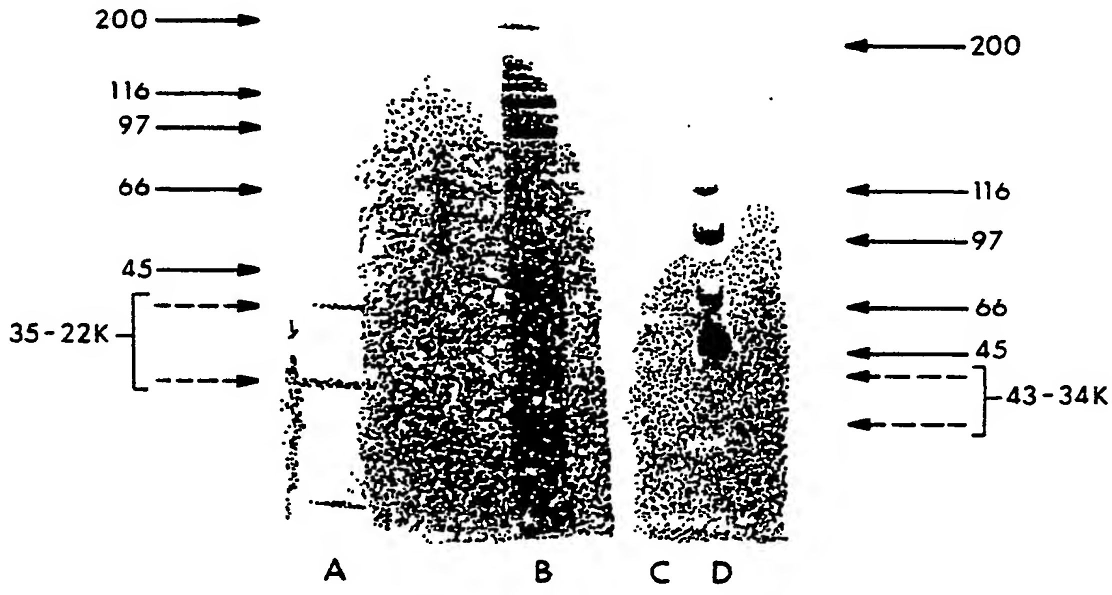



*Fig. 11*




A - Trypsin Digested (35 - 22K)
B - Its Standards
C - Lysozym Digest d (43 - 34K)
D - Its Standards

Fig. 12A

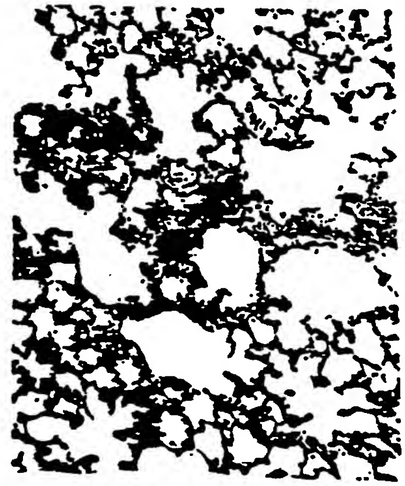


Fig. 12B

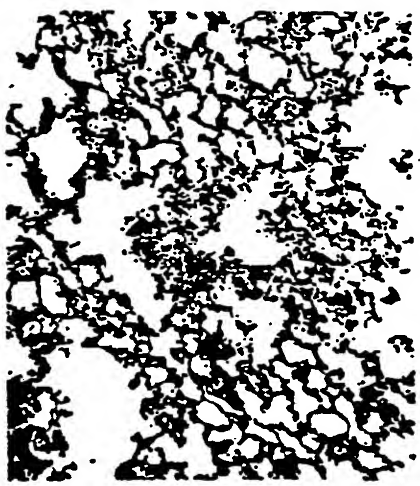


Fig. 12C

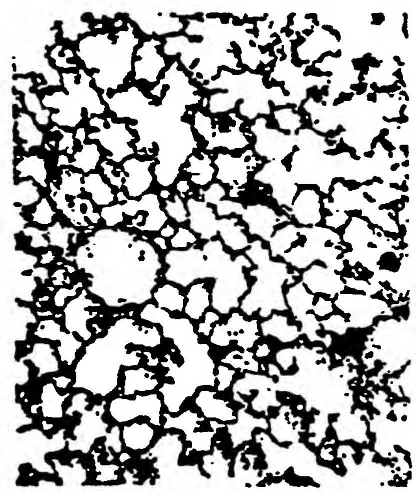


Fig. 12D

HISTOLOGIC APPEARANCE OF MOUSE LUNGS

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/01118

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC
INT. CL.4 C07K 15/04; A61K 37/64; C12Q 1/38; C12P 21/00
U.S. CL. 260/112R; 435/68, 23, 272; 514/2

**II. FIELDS SEARCHED**

Minimum Documentation Searched [4]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 260/112R; 435/68, 885, 883, 852, 23, 177, 180, 272 514/2, 824, 825, 851; 436/531 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [14]

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| Y | N, Chemical Abstract, Vol. 86, Issued 1977, Schiessler et al, "Elastase from human and canine granulocytes,II Interaction with protease inhibitors of animal, plant, and microbial orgin," page 184, col. 1 Abstract No. 85312b. | 1-15 |
| Y | N, Biochem. Biophys. Res. Comm., Vol. 83, No. 2, Issued 1978, Omura et al, "Elasnin, A New Human Granulocyte Elastase Inhibitor Produced by a Strain of Steptomyces," page 704-709. | 1-15 |
| Y | N, Journal Antibiotics, Vol. XXVI NO. 12, Issued 1973, Umezawa et al, "Elastatinal, A New Elastase Inhibitor Produced by Actinomycetes," pages 787-789. | 1-11 |
| Y | N, Am. Rev. Respir. Dis. Vol. 121, Issued 1980 Janoff et al, "Prevention of Elastase-Induced Experimental Emphysema by Oral Administration of a Synthetic Elastase Inhibitor," pages 1025-1029. | 12-15 |
| Y | N, Am. Rev. Respir. Dis. Vol. 127, Issued 1983 Powers, "Synthetic Elastase Inhibitors: Prospects for Use in the Treatment of Emphysema," | 12-15 |

* Special categories of cited documents: [15]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search [2] | Date of Mailing of this International Search Report [2] |
|---|---|
| 30 AUGUST 1985 | 16 SEP 1985 |
| International Searching Authority [1] | Signature of Authorized Officer [20] |
| ISA/US | Garnette D. Draper<br>Garnette D. Draper |

International Application No. PCT/US85/01118

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

| | | |
|---|---|---|
| A | N, Journal Biol. Chem., Vol. 245, No. 12, Issued 1970, "Protein Purification by Affinity Chromatography," pages 3059-3065, Cuatrecasas | 16-18 |

**V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ........., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers ........., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

**VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]**

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.